

Histone H3 gene in the Pacific oyster, *Crassostrea gigas* Thunberg, 1793: molecular and cytogenetic characterisations

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Abstract. The Pacific oyster, *Crassostrea gigas* Thunberg, 1793 ($2n = 20$) is an economically important mollusc species cultured throughout the world. The most frequently used technique for molecular cytogenetic studies is fluorescence *in situ* hybridisation which offers new opportunities for the identification of oyster chromosomes. In oysters, it has been used to locate telomeric sequences, satellite DNA, simple sequence repeats, ribosomal RNA genes, and bacteriophage P1 clones. However, regarding chromosome identification, no study has been done with histone H3 gene. Histone H3 is among the most conserved eukaryotic proteins. Most histone H3 genes are repeatedly organised into clusters, which make them an ideal chromosomal marker. In bivalves, some data exist concerning sequence information but little knowledge is available concerning the physical mapping of histone genes. The histone H3 gene was sequenced in *C. gigas* and phylogenetic analysis revealed that *C. gigas* was more closely related to *Ostrea edulis* Linnaeus, 1758 and species of the genus *Mytilus* Linnaeus, 1758. In *C. gigas*, the histone H3 gene was mapped on two different pairs of chromosomes, one at an interstitial site on the long arm of chromosome pair 4, and the other on the telomeres of the smaller chromosome pair (pair 10). Polymorphism was detected on the telomeres of pair 10, once it was possible to observe single or double signals. Comparative chromosomal mapping should improve our understanding of bivalve genome organisation.

Key words: bivalves, *Crassostrea gigas*, fluorescence *in situ* hybridisation, histone H3 gene, phylogenetics, physical mapping.

INTRODUCTION

The Pacific oyster, *Crassostrea gigas* Thunberg, 1793, is an economically important oyster species cultured throughout the world. This species has a diploid chromosome number of $2n = 20$, and all chromosomes are metacentric. Several banding techniques

were already applied to oyster chromosomes. They are very useful for the identification of individual chromosomes and also of particular regions of chromosomes. “Classical” cytogenetic banding (G-, C- and NOR (nucleolus organiser regions) - banding techniques) was already performed in several species of the genus *Crassostrea* Sacco, 1897

(review in Leitão, Chaves, 2008). Molecular cytogenetic banding was also applied to several species of the genus *Crassostrea* (review in Leitão, Chaves, 2008). RE (restriction endonucleases) - banding (Leitão et al., 2004, 2007; Bouilly et al., 2005; Cross et al., 2005) and FISH (fluorescence *in situ* hybridisation) technique with different kinds of labelled probes were already produced in different species of the genus *Crassostrea*. FISH is a rapid and reliable technique for chromosome identification, gene mapping, localisation of gene expression, and analysis of chromosome rearrangements in a wide variety of genomes. Different probes have already been applied successfully to species of the genus *Crassostrea*: telomeric sequences (Guo, Allen, 1997; Wang, Guo, 2001; Cross et al., 2005), satellite DNA (Clabby et al., 1996; Wang et al., 2001), simple sequence repeats (Cross et al., 2005; Bouilly et al., 2008), ribosomal RNA genes (Zhang et al., 1999; Xu et al., 2001; Cross et al., 2003, 2005; Wang et al., 2004, 2005a), and bacteriophage P1 clones (Wang et al., 2005b).

DNA sequences of nuclear and mitochondrial genes (Boudry et al., 2003; Reese et al., 2008), including repetitive satellite DNA sequences (López-Flores et al., 2004) have been variously used to examine the phylogenetic relationships between oysters.

Histone proteins are the major constituents of the eukaryotic chromatin. The family of histone proteins has been subdivided into the core histones (H2A, H2B, H3, and H4), which form the core particle of the nucleosome, and the linker histones (H1), which are involved in the generation of the higher-order chromatin structure (Thoma et al., 1979). Histone H3 is one of the most conserved eukaryotic proteins (Miller et al., 1993) and is an excellent probe for chromosome mapping as histone genes are usually organised in clusters (Maxson et al., 1983). Histone gene mapping has been

achieved in a few species. In bivalves, little knowledge is available concerning the physical mapping of histone genes. Only five species were studied until now: a mussel (Eirín-López et al., 2002, 2004) and four scallops (Zhang et al., 2007). In Mytilidae, FISH experiments on *Mytilus galloprovincialis* Lamarck, 1819 chromosomes revealed the presence of three pairs of signals in three chromosome pairs with the linker histone H1 probe (Eirín-López et al., 2002), and two pairs of signals in two chromosome pairs with a core histone gene probe (Eirín-López et al., 2004). In Pectinidae, histone H3 gene was clustered at two different loci in the genome of *Patinopecten yessoensis* Jay, 1857 or a single locus in the genome of *Chlamys farreri* Jones et Preston 1904, *Chlamys nobilis* Reeve, 1852, and *Argopecten irradians* Lamarck, 1819 (Zhang et al., 2007). Regarding mollusc molecular phylogenetic studies using histone genes, little information is available. No studies were realised in Ostreidae, but some studies have already been performed in Pectinidae (Puslednik, Serb, 2008), Mytilidae (Eirín-López et al., 2004), Veneridae (Kappner, Bieler, 2006) and in gastropods (Colgan et al., 2000).

In the present work, we partially sequenced the histone H3 gene in *C. gigas* and used that data to ascertain the phylogenetic relationships. We also applied FISH technique with histone H3 gene probe in order to determine its location and distribution in the genome of *C. gigas*.

MATERIAL AND METHODS

Animal material and genomic DNA extraction. DNA was extracted from muscle tissue preserved in 70% ethanol using the Quickgene DNA tissue kit S (Fujifilm Life Science). Tissue was sampled from adult oysters bred at the IFREMER (Institut Français de Recherche pour l'Exploitation de

la Mer) hatchery in La Tremblade (Charente-Maritime, France).

For chromosome preparations, embryos were used. Gametes were collected by strip-spawning sexually mature animals. Fertilized gametes were cultured at 23°C in 150-L fibreglass larval tanks of seawater.

PCR. PCR amplification of the histone H3 gene was realised using a pair of primers (forward: 5'-CGTAAATCCACTGGAGGCAAGG-3'; reverse: 5'-GGATGGCGCACAGGTTGGT GTC-3') designed from the H3 nucleotide sequences of *Pecten jacobaeus* Linnaeus, 1758 and *Mimachlamys varia* Linnaeus, 1758 retrieved from GenBank (AY070153 and AY070154 respectively). The PCR reactions used were the standard ones, except that in the labelling PCR we also used digoxigenin-11-dUTP (Roche Molecular Biochemicals). PCR was performed under the following conditions: 5 min denaturation at 94°C, 30 cycles of 1 min at 94°C, 40 s at 66°C and 1 min at 72°C, and a final extension of 5 min at 72°C. PCR amplification products were subjected to 1.5% agarose gel electrophoresis and stained with ethidium bromide.

Sequencing of histone H3 gene in *Crassostrea gigas*, sequence and phylogenetic analyses. The PCR amplification product was excised from a 1.5% agarose gel and purified using the GeneClean II Kit (QBioGene MP Biomedicals). A band of ~ 300 bp was obtained. The purified PCR product was sequenced in both directions using the primers H3 forward (5'-CGTAAATCCACTGGAGGCAAGG-3') and H3 reverse (5'-GGATGGCGCACAGGTTGGT GTC-3'). The DNA sequence of the histone H3 gene from *C. gigas* was deposited in the GenBank Database with the following accession number: HQ009488. The histone H3 gene sequence in *C. gigas* was analysed

for similarity with other known sequences using the National Centre for Biotechnology Information Blast database tool (<http://www.ncbi.nlm.gov/Blast/>). CLC Sequence Viewer version 6.2 (<http://www.clcbio.com>) was used to align the sequence with other histone H3 gene sequences and for phylogenetic reconstruction. A total of 42 histone H3 gene sequences were used for phylogenetic analysis (Table 1). Distance-tree was constructed with the Neighbour-Joining (NJ) algorithm (Saitou, Nei, 1987), 100 bootstrap replicates. Bootstrap values lower than 50% were not shown.

Chromosome preparations. Chromosome preparations were made as described in Bouilly et al. (2008). Briefly, 6 hours old embryos were treated with 0.005% colchicine in seawater for 25 min. A hypotonic shock was applied for 10 min in 0.9% sodium citrate. Then, embryos were fixed in 3:1 absolute ethanol-acetic acid solution. Embryos cells were dissociated in 50% acetic acid and the suspension obtained was dropped onto slides heated at 42°C and air-dried.

Fluorescence in situ hybridisation (FISH). Chromosome spreads were pretreated with 0.005% pepsin in 10 mM HCl at 37°C for 5 min and air-dried. Slides were then fixed with formaldehyde as described in Chaves et al. (2002). Briefly, slides were rinsed twice in phosphate-buffered saline (PBS) for 5 min, and after incubation in 1% formaldehyde in PBS, for 20 min at room temperature, they were washed twice in PBS for 5 min. The slides were then dehydrated in a chilled ethanol series (70%, 90%, and 100%; 5 min each) and air-dried. Slides were aged overnight at 65°C, and then, dehydrated in 100% ethanol at -20°C for 3 min. Chromosomes preparations were denaturated at 72°C in 70% formamide in 2 × SSC for 2 min, followed by dehydration in 70%, 90%, and 100% chilled ethanol, for 5 min each. The probe was denaturated at 80°C for

Table 1. List of histone H3 gene sequences in different molluscan species used for the phylogenetic analysis with GenBank accession numbers.

Phylum	Class	Superorder	Order	Family	Species	Accession number
Mollusca	Bivalvia	-	Ostreoida	Ostreidae	<i>Crassostrea gigas</i> Thunberg, 1793	HQ009488
					<i>Ostrea edulis</i> Linnaeus, 1758	AY070151
			Mytiloida	Mytilidae	<i>Mytilus californianus</i> Conrad, 1837	AY267745
					<i>Mytilus chilensis</i> Hupé, 1854	AY267746
					<i>Mytilus edulis</i> Linnaeus, 1758	AY267749
					<i>Mytilus galloprovincialis</i> Lamarck, 1819	AY267748
					<i>Mytilus trossulus</i> Gould, 1850	AY267747
			Limoida	Limidae	<i>Limaria hemphilli</i> Hertlein & Strong, 1946	EU379502
					<i>Ctenoides annulata</i> Lamarck, 1819	EU379493
			Pectinoida	Pectinidae	<i>Laevichlamys irregularis</i> Sowerby, 1842	EU379537
					<i>Coralichlamys madreporarum</i> Sowerby II, 1842	EU379505
					<i>Patinopecten caurinus</i> Gould, 1850	FJ263662
					<i>Chlamys islandica</i> Müller, 1776	FJ263666
					<i>Argopecten gibbus</i> Linnaeus, 1758	EU379496
					<i>Euvola ziczac</i> Linnaeus, 1758	EU379538
					<i>Pecten jacobaeus</i> Linnaeus, 1758	AY070153
					<i>Amusium pleuronectes</i> Linnaeus, 1758	EU379523
					<i>Nodipecten subnodosus</i> Sowerby, 1835	EU379535
					<i>Mimachlamys varia</i> Linnaeus, 1758	AY070154
					<i>Aequipecten opercularis</i> Linnaeus, 1758	EU379516
					<i>Leptopecten bavayi</i> Dautzenberg, 1900	EU379487
			Veneroida	Veneridae	<i>Antigona lamellaris</i> Schumacher, 1817	DQ184882
					<i>Meretrix meretrix</i> Linnaeus, 1758	FJ429106
				Glauconomidae	<i>Glauconome chinensis</i> Gray, 1828	DQ184899
				Pharidae	<i>Ensis ensis</i> Linnaeus, 1758	AY070159
					<i>Phaxas pellucidus</i> Pennant, 1777	DQ280006
				Semelidae	<i>Abra prismatica</i> Montagu, 1808	AY070160
				Carditidae	<i>Cardita calyculata</i> Linnaeus, 1758	AY070156
			Myoida	Pholadidae	<i>Nettastomella japonica</i> Yokoyama, 1920	AB439267
				Myidae	<i>Mya arenaria</i> Linnaeus, 1758	AY070164
	Gastropoda	Caenogastropoda	Sorbeoconcha	Turridae	<i>Turris babylonia</i> Linnaeus, 1758	EU015786
				Terebridae	<i>Cinguloterebra cf. fujitai</i> Kuroda & Habe, 1952	EU015832
				Conidae	<i>Etrema</i> sp.	EU015800
					<i>Tritonoturris</i> sp.	EU015823
					<i>Raphitoma</i> sp.	EU015813
					<i>Conus gauguini</i> Richard & Salvat, 1973	EU015856
				Littorinidae	<i>Littorina littorea</i> Linnaeus, 1758	DQ093507
		Vetigastropoda	-	Stomate-llidae	<i>Stomatella</i> sp.	AY923978
				Trochidae	<i>Gibbula zonata</i> Wood, 1828	AY923977
				Turbinidae	<i>Astraea undosa</i> Wood, 1829	AY923980
					<i>Homalopoma maculosa</i> Pease, 1868	AY923982
				Haliotidae	<i>Haliotis midae</i> Linnaeus, 1758	AY923957

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      20      40      60
c g t a a a t c c a c t g g a g g c a a g g c t c c a c g t a a a c a a c t g g c t a c c a a g g c c g c c c g t a a g a g c g c c c c a g c
      80      100      120      140
c a c t g g t g g a g t c a a g a a c c c c a c a g a t a c a g g c c c g g a a c c g t c g c t c t c c g t g a g a t c a g g a g a t a c c
      160      180      200
a g a a a g c a c a g a g t t g c t c a t c a g g a a a t t g c c c t t c c a g c g t c t g g t c c g t g a g a t t g c t c a g g a c t t c
      220      240      260      280
a a g a c t g a t c t g c g a t t c c a g a g c t c c g c c g t c a t g g c t c t c c a g g a a g c c a g c g a a g c t t a c c t t g t t g g
      300
a c t c t t t g a g g a c a c c a a c c t g t g c g c c a t c c

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Fig. 1. Histone H3 gene sequence from *Crassostrea gigas*.

10 min and cooled immediately. Hybridisation solution containing the denaturated probe was dropped onto the denaturated spreads, the slides were covered and kept overnight in a humid chamber at 37°C. After hybridisation, the slides were washed at 37°C once in 2 × SSC for 5 min, then twice in 50% formamide in 2 × SSC for 5 min, and once in 2 × SSC for 5 min. The blocking agent was 3% BSA, for 10 min at room temperature. The digoxigenin-labelled-probe was detected with anti-digoxigenin-rhodamine fab fragments (Roche Molecular Biochemicals). The slides were counterstained with DAPI and mounted in Vectashield (Vector Laboratories).

Chromosome observation. Chromosomes were observed with a Zeiss Imager.Z1 microscope coupled with an Axiocam digital camera and a Zeiss LSM Image Browser software. Digitised photos were prepared for printing in Adobe Photoshop (Version 9.0); contrast and color optimisation were the functions used and all affected the whole of the image equally.

RESULTS AND DISCUSSION

Molecular characterisation. A histone H3 gene sequence in *C. gigas* with a size of approximately 300 base pairs was isolated and sequenced. A contig was produced using Vector

NTI Advance 10. The length of this contig was 326 bp. Primers were aligned with the contig and unalignable regions were excluded from phylogenetic analysis. These included positions 1 to 3 and 320 to 326. The sequence, after analysis, was 316 base pairs long (Fig. 1). The analysis of *C. gigas* H3 gene sequence using NCBI Blast database tool showed a high similarity with H3 gene sequence in different mollusc species. For example, *C. gigas* H3 gene had 87% similarity with *O. edulis* H3 gene (AY070151). The GC content (56.3%) was higher than the AT content (43.7%). The histone H3 gene sequence from *C. gigas* and other histone H3 gene sequences were reduced to 305 bp for phylogenetic analysis to have a good alignment with the 41 histone H3 gene sequences selected from GenBank Database. The NJ tree separated the species into two clades (Fig. 2). The first clade was subdivided into two groups, one constituted of 20 Bivalvia (12 Pectinidae, 5 Mytilidae, 2 Ostreidae and 1 Limidae) all from the subclass Pteriomorpha, and the other one of 5 Gastropoda (corresponding to the superorder of Vetigastropoda), and 7 Bivalvia (all Heteroconchia: 4 Veneroidea, 2 Myoidea, except one which was Pteriomorpha: 1 Limoida).

In bivalves, phylogenetic relationships with histone H3 gene sequences have already been studied in Pectinidae (Puslednik, Serb,

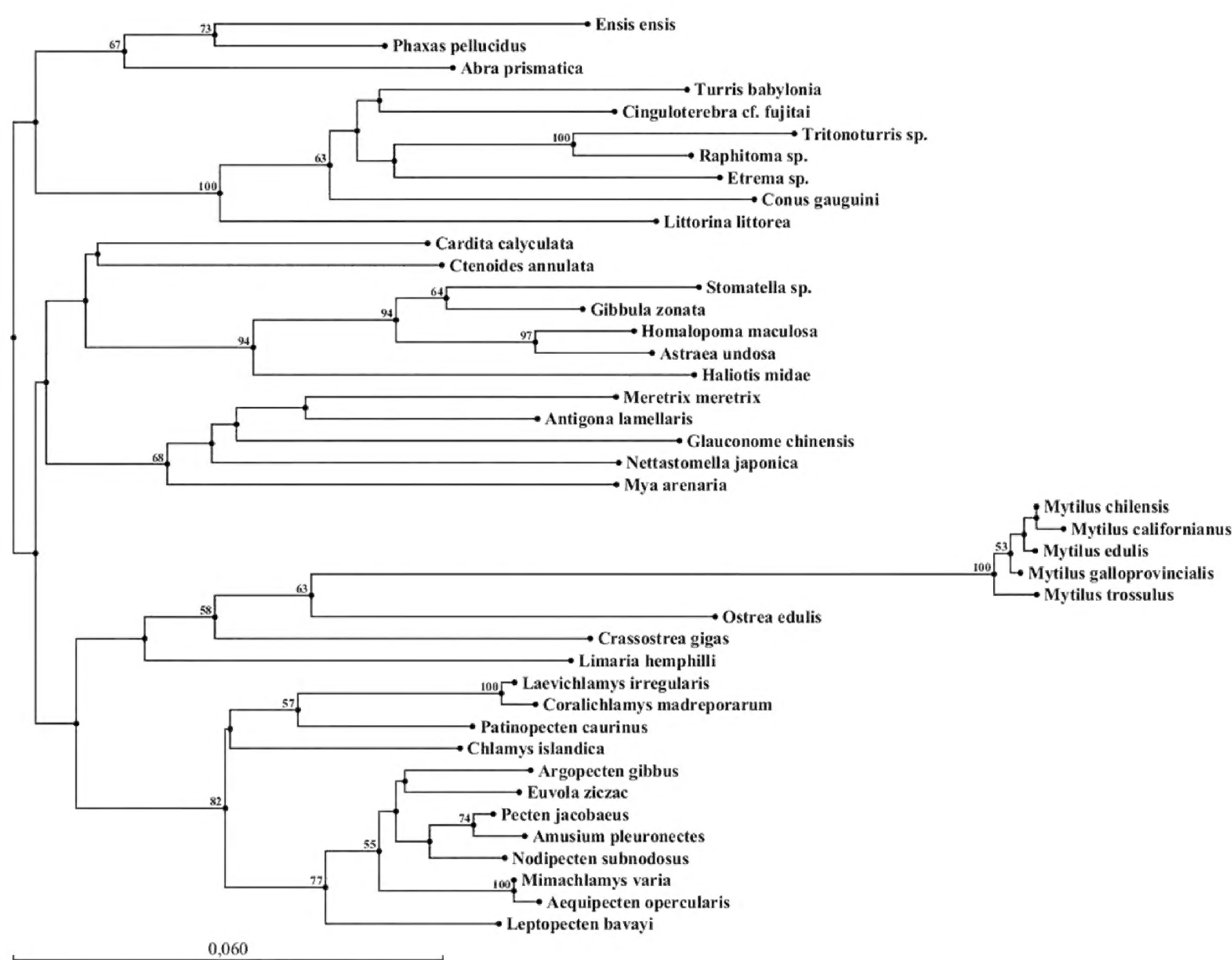


Fig. 2. The phylogenetic relationships among different molluscan species, as analysed by the Neighbour-Joining method. Bootstrap values over 50% are written above the branches. Bar = Substitutions/Site.

2008), in Veneridae (Kappner, Bieler, 2006), and in Mytilidae (Eirín-López et al., 2004). Unfortunately, little knowledge was available concerning Ostreidae as until now only two sequences of histone H3 gene were available in oysters, the one from *O. edulis* and the one from *C. gigas* (this study). Ostreida, Mytiloida, Pectinoida and Limoida are from the same subclass: Pteriomorphia, therefore it was expected to find them in the same clade. The other Limoida (*Ctenoides annulata* Lamarck, 1819) was found in another clade which was unexpected, but it was also not supported by

a significant bootstrap value. The closest relative of this Limoida was a Veneroida. This Limoida was also closest to a group of Vetigastropoda species (representatives of another class, Gastropoda). The bootstrap values were not significant, thus, no relationships between these different species can be established. *C. gigas* was more closely related to *O. edulis* which is a predictable result as they both belong to the same family Ostreidae, and also to different Mytilidae species: *Mytilus californianus* Conrad, 1837, *M. chilensis* Hupé, 1854, *M. edulis* Linnaeus,

1758, *M. galloprovincialis*, and *M. trossulus* Gould, 1850. However, *O. edulis* seems to be more closely related to species of the genus *Mytilus* than to *C. gigas* which is a species from the same family. The reason for this peculiar result could be the short length of the sequence (only around 300 bp) used for the reconstruction. Eirín-López et al. (2004) studied 5 Mytilidae, the same sequences that we selected in GenBank Database. The results observed by Eirín-López et al. (2004) were quite similar to the ones observed in this study as they showed that the 5 *Mytilus* species: *M. californianus*, *M. edulis*, *M. chilensis*, *M. galloprovincialis* and *M. trossulus* were clustered together.

According to the phylogenetic tree some closely related species were not clustered together. The partial sequence of histone H3 gene seems to be too short to give a good resolution in phylogenetic reconstructions. Indeed, some groups were not supported by a significative bootstrap value, and thus, H3 gene did not allow reconstructing an accurate phylogeny as the different clusters not always correspond to the taxonomical (family level or class level) groups.

Cytogenetic characterisation. We examined 80 good chromosome spreads from oyster embryos. Our results showed that the histone H3 gene was mapped at two different *loci* in the genome of *C. gigas* (Fig. 3). The histone H3 gene was located at an interstitial site (about half-way from the centromere to the telomeres) on the long arm of chromosome pair 4, and on the telomeres of the smaller chromosome pair (pair 10). Polymorphism was detected on the telomeres of pair 10. On the 80 metaphases that were examined, 16 presented single signals in both chromosomes (20%) (Fig. 3, A-B), 29 showed double signals (36%) (Fig. 3, C-D), and 35 had a single signal on one chromosome and a double signal on the

homologue (44%) (Fig. 3, E-F). The histone H3 gene is a qualified chromosomal marker.

In other bivalve species, histone genes were also localised in one, two or three loci. In Mytilidae, FISH results on *Mytilus galloprovincialis* chromosomes located core histone genes at two loci in two different chromosome pairs (Eirín-López et al., 2004), and the linker histone H1 probe at three loci in three chromosome pairs (Eirín-López et al., 2002). In both experiments, the hybridisation signals mapped the histone genes at telomeric chromosomal positions or interstitial positions. In Pectinidae, histone H3 gene was mapped at two loci in the genome of *Patinopecten yessoensis* or a single locus in the genome of *Chlamys farreri*, *Chlamys nobilis*, and *Argopecten irradians* (Zhang et al., 2007). Positions were also interstitial or telomeric as in our experiment. In Mytilidae and Pectinidae studies, no polymorphism was reported. Histone gene clusters were rather conservative in chromosome location in most cases where closed species have been analysed (Hankeln et al., 1993; Ranz et al., 2003; Cabrero et al., 2009). Chromosomal localisation of histone H3 gene should be determined in other *Crassostrea* species for oyster comparative studies. Histone H3 gene in *O. edulis* was characterised molecularly (Giribet, Distel, 2003), but no cytogenetic studies were realised with this sequence.

Major ribosomal DNA genes (Xu et al., 2001; Wang et al., 2004), NORs (Thiriot-Quiévreux, Insua, 1992) and C-bands (Bouilly et al., 2008) were also localised on the telomeres of long arm of pair 10 in *C. gigas*. During mitosis, the major ribosomal genes are the nucleotidic component of the NORs (Goessens, 1984), therefore, the chromosomal sites of major ribosomal gene clusters correspond to NOR sites. NOR sites were already detected to colocalise with C-banded

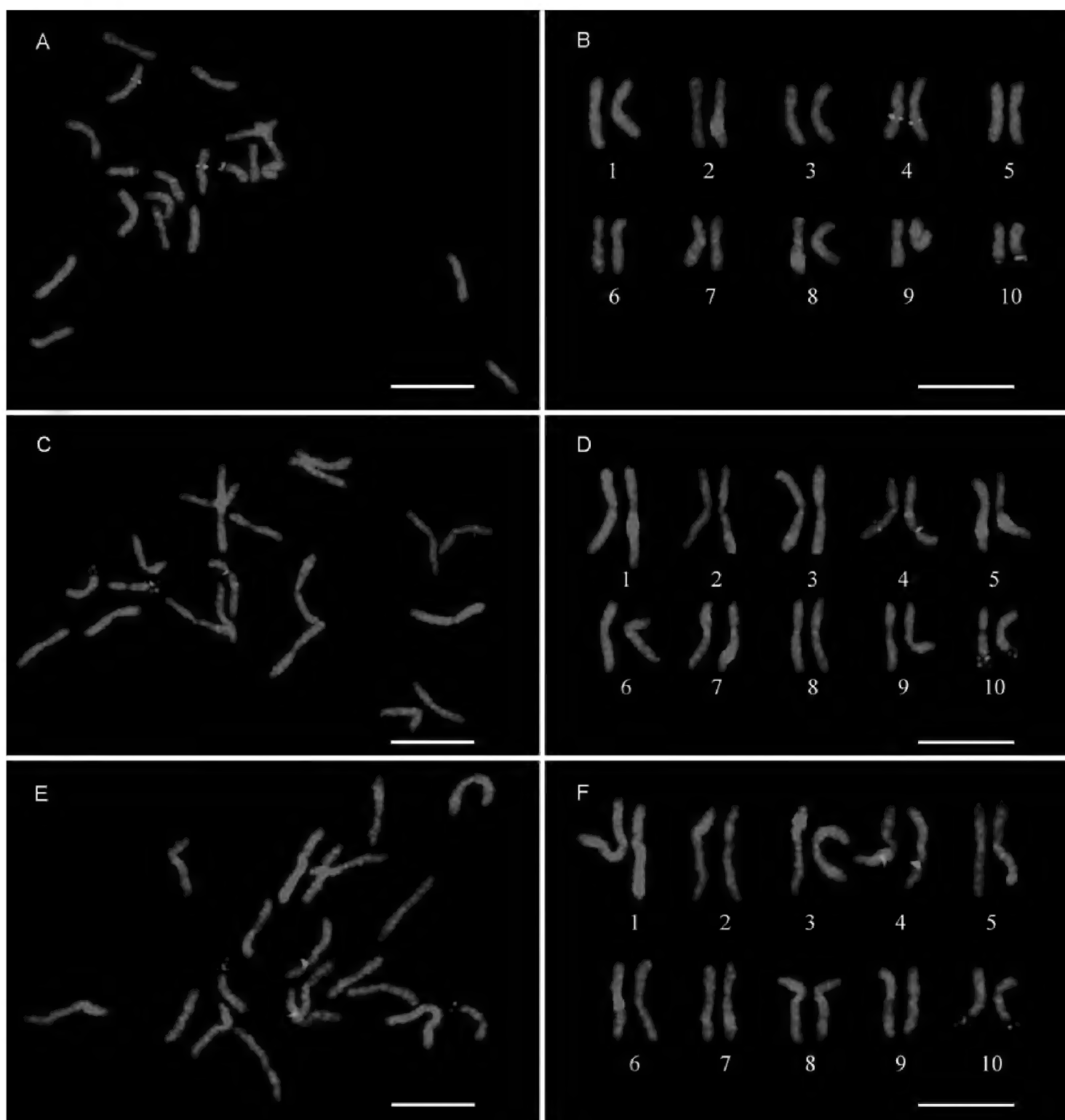


Fig. 3, A-F. FISH with histone H3 gene probe applied on chromosomes of *Crassostrea gigas* (in red). The chromosomes were contrasted with DAPI (blue). **A** - a metaphase cell with single/single signals on telomeres of pair 10, and **B** - its corresponding karyotype. **C** - a metaphase cell with double/double signals on telomeres of pair 10, and **D** - its corresponding karyotype. **E** - a metaphase cell with single/double signals on telomeres of pair 10, and **F** - its corresponding karyotype. Bars = 10 μ m.

heterochromatic region in an insect *Bradysia hygida* Sauaia et Alves, 1968 (Gaspar et al., 2002). In salmonids, the chromosomal location of the major histone cluster was adjacent or close to regions with C+ heterochromatin (Pendás et al., 1994). Histone genes were already found to be associated with 5S rDNA in two Crustacean species, *Artemia salina*

Linnaeus, 1758 (Andrews et al., 1987) and *Asellus aquaticus* Linnaeus, 1758 (Barzotti et al., 2000). Multicolour-FISH will be of great interest to check if the major and minor rDNA genes are colocalised or not with the histone H3 genes in *C. gigas*.

In conclusion, histone H3 gene is a useful karyotypic marker which can be used in

oyster cytotaxonomy. Our data support that molecular and cytogenetic characterisations of histone H3 gene in other oyster and bivalve species will be useful for comparative studies, evolutionary and phylogenetic studies, and for understanding genome organisation in oysters.

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